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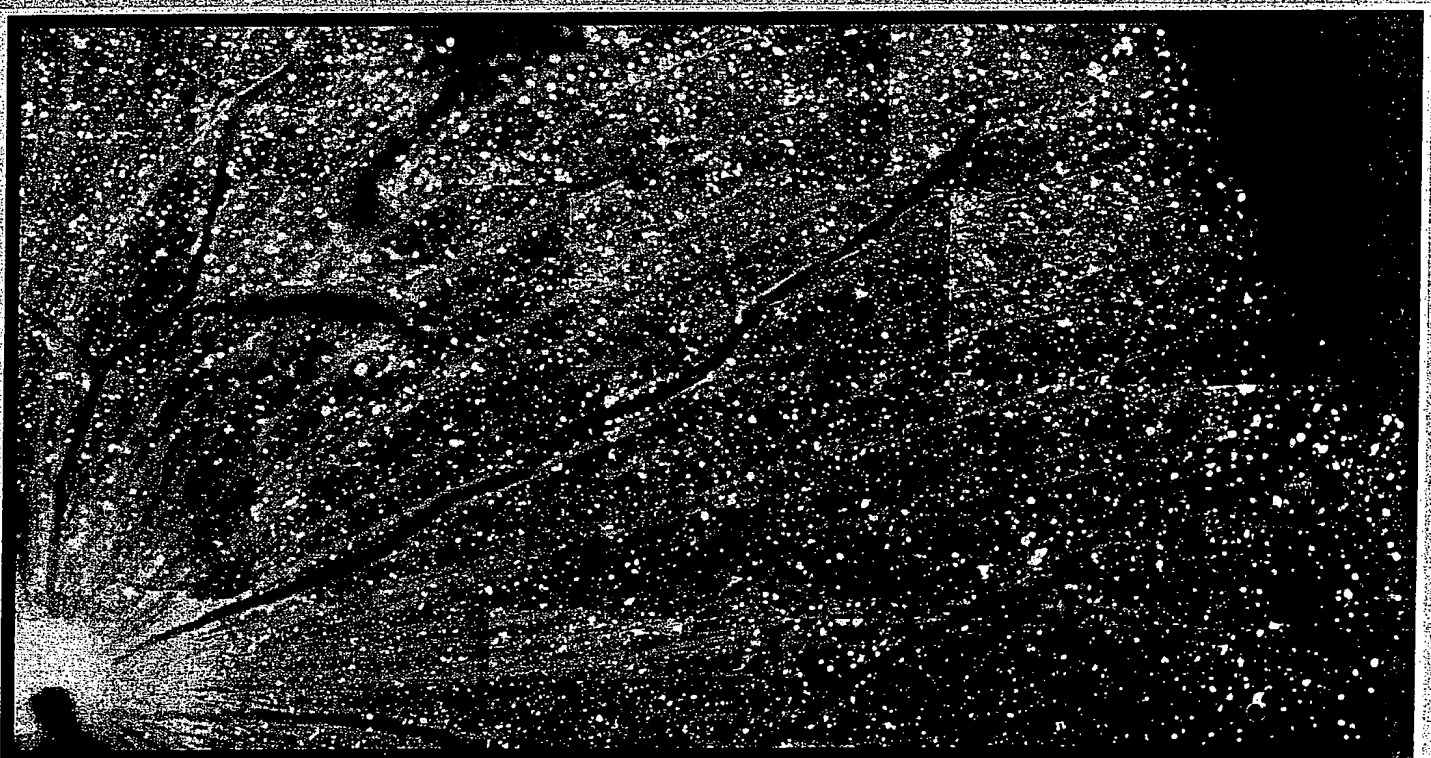

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Cover photo. Retrogradely labeled normal, noninjured retinal ganglion cells. See Yoles and Schwartz, 1998, **153**, 1–7.

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Degeneration of Spared Axons Following Partial White Matter Lesion: Implications for Optic Nerve Neuropathies

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Neuroprotective therapy is a relatively new development in the approach to the treatment of acute and chronic brain damage. Though initially viewed in the framework of acute CNS injuries, the concept was recently extended to include chronic injuries, in which at any given time there are some neurons in an acute phase of degeneration coexisting with others that are healthy, marginally damaged, or dead. The healthy neurons and those that are only marginally damaged are the potential targets for neuroprotection. For the development of neuroprotective therapies, it is essential to employ an animal model in which the damage resulting from secondary degeneration can be quantitatively distinguished from primary degenerati n. This is of particular relevance when the site of the damage is in the white matter (nerve fibers) rather than in the gray matter (cell bodies). In the present work we reexamine the concepts of secondary degeneration and neuroprotection in white matter lesions. Using a partial crush injury of the adult rat optic nerve as a model, we were able to assess both primary and secondary nerve damage. We show that neurons whose axons were not damaged or only marginally damaged after an acute insult will eventually degenerate as a consequence of their existence in the degenerative environment produced by the injury. This secondary degeneration does not occur in all of the neurons at once, but affects them in a stepwise fashion related to the severity of the damage inflicted. These findings, which may be applicable to the progression of acute or chronic neuropathy, imply that neuroprotective therapy may have a beneficial effect even if there is a time lag between injury and treatment. © 1998 Academic Press

Key Words: **neuroprotection; secondary degeneration; optic nerve; CNS trauma.**

INTRODUCTION

It is now widely accepted that neuronal loss following nerve injury is greater than might be expected from the severity of the injury and that this is attributable to a variety of processes leading collectively to secondary degeneration. Attempts have been made to identify the mediators of secondary degeneration, with the aim of neutralizing them and/or their effects. The term neuroprotection was recently applied to describe this approach (9, 10, 28). "Neuroprotection" refers to the protection of neurons which, following acute nerve insult, do not sustain direct injury, but are adjacent to or surrounded by a damaged milieu and will consequently undergo "secondary degeneration" unless adequately treated (9, 10, 28). In general, this is true for both gray and white matter. Injury of white matter leads inevitably to degeneration of fibers that sustained the primary insult, and even if their cell bodies are successfully protected there will be no recovery of function unless regeneration can be induced to occur (26). In the case of primary injury of gray matter, the damaged cell bodies rapidly die. Thus, in either case, in terms of functional protection, neuroprotective therapy would at best imply only protection of those neurons that either escaped the primary insult or were marginally damaged but retained their functional integrity.

Studies carried out in animal models have led to the identification of some of the agents that mediate damage to neurons that escaped primary injury (3, 9, 10, 16, 17, 19, 24, 28). In order to document their effects on these initially spared neurons, one must first be able to verify that secondary degeneration has in fact taken place, i.e., to distinguish between neurons that have degenerated as a result of the primary injury and those that have degenerated as a result of a secondary process.

In principle, loss of neurons can be documented by evaluation of behavioral tasks (2, 6), assessment of electrophysiological parameters (32, 33), imaging techniques (11, 14), or morphological criteria (4).

In the present study we demonstrate the occurrence of secondary degeneration after acute axonal (white

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matter) injury in the central nervous system (CNS). Specifically, we show that primary partial axotomy of adult rat optic nerve is followed by an immediate degeneration of the neurons that suffered direct damage, while the rest retain their anatomical integrity; with time, however, there is a stepwise loss of neurons that escaped the initial injury or were only marginally damaged by it. Thus, even if no other injury-inducing factors are present, intact or marginally damaged neurons will gradually degenerate if they are immersed in a degenerative environment. We further show that although the occurrence of such secondary degeneration is not directly dependent on the primary lesion itself, its extent is related to the severity of the primary damage.

METHODS

Animals

Animal utilization was in accordance with the ARVO resolution on the use of animals in research. Adult male Sprague–Dawley (SPD) rats weighing 300–350 g from the Weizmann Institute of Science animal house were anesthetized with Vetalar (ketamine, 50 mg/kg) and Rompun (xylazine, 0.5 mg/kg) administered intraperitoneally. Prior to tissue excision animals were deeply anesthetized by an overdose of sodium pentobarbitone (170 mg/kg, intraperitoneally).

Crush Injury

Using a binocular operating microscope, lateral canthotomy was performed in the right eyes of anesthetized rats. The conjunctiva was incised laterally to the cornea, the retractor bulbi muscle separated, and the optic nerve exposed. Using calibrated cross-action forceps, a moderate, mild, or very mild crush injury (1, 8, 32, 33) was inflicted on the nerve 2 mm from the eyeball, taking special care not to interfere with the retinal blood supply. Use of these forceps makes it possible to inflict reproducible lesions of differing severity by varying the number of screw revolutions attached to the forceps (1, 8).

Morphometric Analysis of Primary and Secondary Degeneration

Primary damage was measured following the immediate postinjury application of the neurotracer dye 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes, Europe BV) distally to the site of injury. Only axons that are intact are capable of transporting the dye back to their cell bodies; therefore, the number of labeled cell bodies is a measure of the number of axons that survived the primary insult. Secondary degeneration was also measured by the application of the dye distally to the injury site, but at various time points after the primary lesion was inflicted. Application of the dye in this way (i.e., distally to the site of the primary crush and after different periods of time) ensures that only axons that survived both primary and secondary degeneration will be counted. This approach makes it possible to differentiate between undamaged neurons and injured neurons with still-viable retinal ganglion cells, since only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Thus, the number of labeled ganglion cells reliably reflects the number of still-functioning neurons.

Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of the fluorescent lipophilic dye 4-Di-10-Asp (29) were deposited at the site of the newly formed axotomy. Noninjured optic nerves were similarly labeled at approximately the same distance from the globe. One week after dye application the animal was given a lethal dose of pentobarbitone (170 mg/kg). The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled ganglion cells by fluorescence microscopy. Retinal ganglion cells were counted as described under Results. It should be noted that this method of labeling does not yield an absolute number of intact neurons; it does, however, allow a reliable quantitative comparison between treated and untreated nerves. Calculation of the number of labeled neurons relative to the number of neurons of control noninjured nerves and of injured nerves labeled immediately after the primary damage provides a quantitative measure of the total degeneration and of secondary degeneration, respectively.

Stereotactic Dye Application Prior to Injury

This staining technique was performed in order to assay the total number of surviving ganglion cells of injured and intact fibers in the retina. Two weeks prior to crush injury, rats were deeply anesthetized and placed in a small stereotactic instrument. The skull was exposed and kept dry and clean using 3% hydrogen peroxide. Bregma was identified and marked. A hole was drilled above the superior colliculus of the left hemisphere (6 mm behind and 1.2 mm in front of the bregma). Using a stereotactic measuring device and Hamilton injector we injected Fluoro-Gold (5% in water, Fluorochrome Inc.; 2 μl/2 min for each point) at 3 points in the superior colliculus (at depths of 3.8, 4, and 4.2 mm from the bony surface of the brain). After completion of the injection the wound was sutured. Two weeks later the animal's right optic nerve was crush-

injured. After a further 2 weeks some of the animals were subjected to a complete axotomy, which was inflicted distally to the primary injury. Three or 7 days later the animals were given a lethal dose of pentobarbitone (170 mg/kg), and their retinas were detached from the eyes and examined for stained ganglion cells by fluorescence microscopy. Retinas of noninjured nerves were also stereotactically labeled and their retinal ganglion cells counted.

RESULTS

Quantitative Proof of Secondary Neuronal Death

Primary damage and secondary degeneration were assessed morphometrically as described under Methods. Briefly, the neurotracer dye was applied distally to the lesion site immediately (to assess primary damage) or 2 and 4 weeks later (to assess secondary degeneration). To obtain the number of labeled retinal ganglion cells (RGCs) in each retina, five fields of identical size were randomly selected and their RGCs counted and averaged. Figure 1 shows the numbers of RGCs/mm^2 in retinas of nerves with moderate or mild injuries, immediately and 2 and 4 weeks after the injury. As shown, the extent of primary damage (approximately 58 and 27% in moderate and mild injuries, respectively) is a function of the severity of the insult. To examine whether secondary degeneration is a self-limiting process, we also examined animals at 9 or 27 weeks following the injury and included those with very mild injuries as well. It was interesting to note that the secondary degeneration also reflects the severity of the primary insult; the more severe the primary insult, the higher the percentage of secondary degeneration, as calculated by the following equation: [number of spared neurons (primary-secondary)/number of neurons spared (primary)] × 100%. In both mild and very mild injuries the secondary degeneration was self-limiting (Fig. 2).

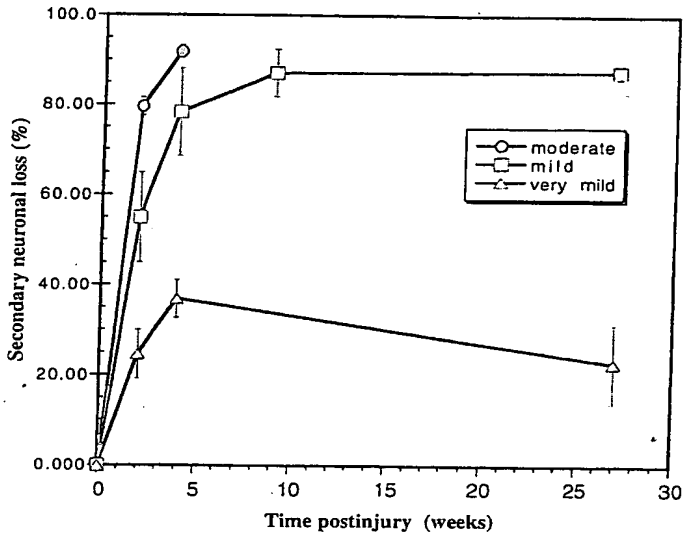

FIG. 2. Secondary degeneration as a function of time in rat optic nerves following injuries of varying severity. The figure shows secondary degeneration, defined and calculated as the percentage of neurons that degenerated subsequent to the primary damage. Three groups of injured nerves were included in this comparison: moderately injured, mildly injured, and very mildly injured. Labeling and analysis at the indicated time points after injury were performed as described in the legend to Fig. 1. Note that a steady state was reached after mild or very mild injury but not after moderate injury. A minimum of three animals and in some groups six or eight animals, at all time points and in each group, were examined.

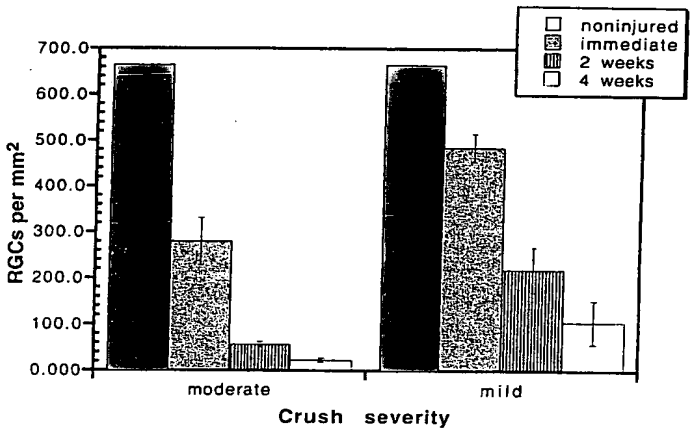

FIG. 1. Progressive loss of neurons left intact after axonal injuries of varying severity. 4-Di-10-Asp was applied to rat optic nerves at different times after the nerves had been subjected to crush injuries of moderate or mild severity. One week later the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from five fields (located at approximately the same distance from the optic disk) in each retina were counted and their average number per mm^2 was calculated. Bars represent mean values ± SEM of retinas of noninjured nerves (n = 2); moderately injured nerves that were retrogradely labeled immediately (n = 2), 2 weeks (n = 6), and 4 weeks (n = 8) after injury; mildly injured nerves retrogradely labeled immediately (n = 3), 2 weeks (n = 3), and 4 weeks (n = 4) after injury.

Validation of the Morphometric Method of Quantification

In order to apply a dye distally to the site of the lesion a second lesion is needed. It was therefore important to know whether the second axotomy would result in a further loss of labeled RGCs up to the time (7 days later) of retinal excision for RGC counting. If so, we would expect to find fewer labeled RGCs in the retinas of twice-injured nerves than in retinas of animals subjected only to primary insult. All RGCs were labeled prior to the primary crush injury by stereotactic injection of the dye into the brain, as described under Methods, and 2 weeks later a moderate crush injury was inflicted. Two weeks later some animals were subjected to an additional axotomy, similar to that

TABLE 1

Surviving Retinal Ganglion Cells Following Crush Injury and with or without Second Axotomy

Days post-axotomy	Crush injury no axotomy (RGCs/mm^2 ± SEM)	Crush injury + axotomy (RGCs/mm^2 ± SEM)
3	1015.5 ± 60.5 (n = 2)	974 ± 10.54 (n = 3)
7	918.5 ± 66.5 (n = 2)	971.67 ± 38.8 (n = 3)

Note. Normal retina (no crush, no axotomy), 2908.5 ± 110.5 RGCs/mm^2. Fluoro-Gold was injected stereotactially into the superior colliculus of naive animals. Two weeks later the nerves were exposed unilaterally and moderately crushed. After a further 2 weeks, some of the animals were reanesthetized and the same optic nerves were reexposed and subjected to a second lesion in the form of a transection distal to the primary insult. Retinas were excised from all animal 3 or 7 days later. Results are expressed as the mean number of retinal ganglion cells per mm^2 ± SEM. One-way analysis of variance revealed no differences between animals that were subjected to a second lesion and those that were not (P = 0.57).

performed for dye application. Retinas were excised from all animals 3 or 7 days later and their RGCs were counted. As shown in Table 1, the second lesion caused no further significant loss of labeled RGCs after 3 or 7 days. This finding implies that although the second axotomy causes death of the newly axotomized neurons, it takes more than a week for their cell bodies to disappear. This experiment therefore confirms the validity of the morphometric analysis involving distal dye application.

Differential Susceptibilities of Neurons to the Primary Insult and to Secondary Degeneration as a Function of Their Topography

To find out whether the vulnerability of the axons to primary or secondary degeneration is affected by their topography, we mapped the retinal ganglion cell distribution in retinas of noninjured nerves and of injured nerves 2 and 4 weeks after moderate crush. The distribution patterns in retinas of injured optic nerves are shown in Fig. 3. Close comparison between the results for noninjured nerves and for injured nerves at 2 and 4 weeks after moderate crush revealed that in the former, RGC density was very similar in all tested zones except for a slight but nonsignificant tendency to decrease toward the retinal periphery. Following secondary degeneration, this difference became more pronounced and was significant (Figs. 4 and 5). These findings raised the question of whether topographical selectivity is triggered by the primary insult itself or by subsequent secondary events. To address this question, we analyzed retinal ganglion cell topography immediately after injury. The results showed that immediately after a primary insult, even of mild severity, there is already a preferential loss of neurons whose ganglion cells are located in the retinal periphery (Fig. 6). It therefore seems that the topographical selectivity is a reflection of the susceptibility of the axons to the injury, the most vulnerable axons being those whose cell bodies are located peripherally. All subsequent degeneration is dictated by the topographical distribution of the acutely injured neurons.

DISCUSSION

In this study we show that axons that escaped a primary axonal injury will nevertheless degenerate because of their proximity to axons degenerating due to the primary insult. We further show that the secondary degeneration is self-limiting, and that its extent is a function of the severity of the primary insult.

Secondary degeneration following acute or chronic lesions or disorders of the CNS (7, 13, 19, 21, 27, 30) is now widely recognized as an integral part of the response to any acute CNS event, such as trauma or ischemia. Intensive research efforts have therefore been directed toward uncovering the mediators of secondary degeneration and seeking ways to neutralize them, or their effects, or the receptors with which they interact (3, 4, 15, 18, 31, 34). These studies have guided the way to neuroprotective therapy.

An obstacle to progress in neuroprotection can be the experimental model (12). This issue is of particular relevance in white matter lesions such as axotomies and chronic neuropathies. We show here that partial lesion of the optic nerve leads to measurable secondary degeneration. Other characteristics of the secondary degeneration observed in our study were its gradual, stepwise progression and self-limiting. This implies that neuroprotective therapy might need to be administered more than once and to be properly synchronized with the relevant physiological events, as protection given at time zero may not be sufficient for protection against delayed onset of degeneration. It should, however, be taken into account that among the secondarily degenerating fibers that might be fibers that were victims of the primary insult and though phenotypically remained undamaged, nevertheless became more vulnerable to the hostile environmental conditions created by the primarily degenerating fibers.

It should be noted that, in principle, secondary degeneration following axonal injury may start either in the axons of spared neurons or in their cell bodies. The process may occur in the following manner: after the primary insult, injured neurons release toxic substances that spread to neighboring neurons. As a consequence the latter neurons, despite having escaped the primary injury, undergo secondary degeneration, releasing additional toxic substances into their environment and injuring more neurons (tertiary damage),

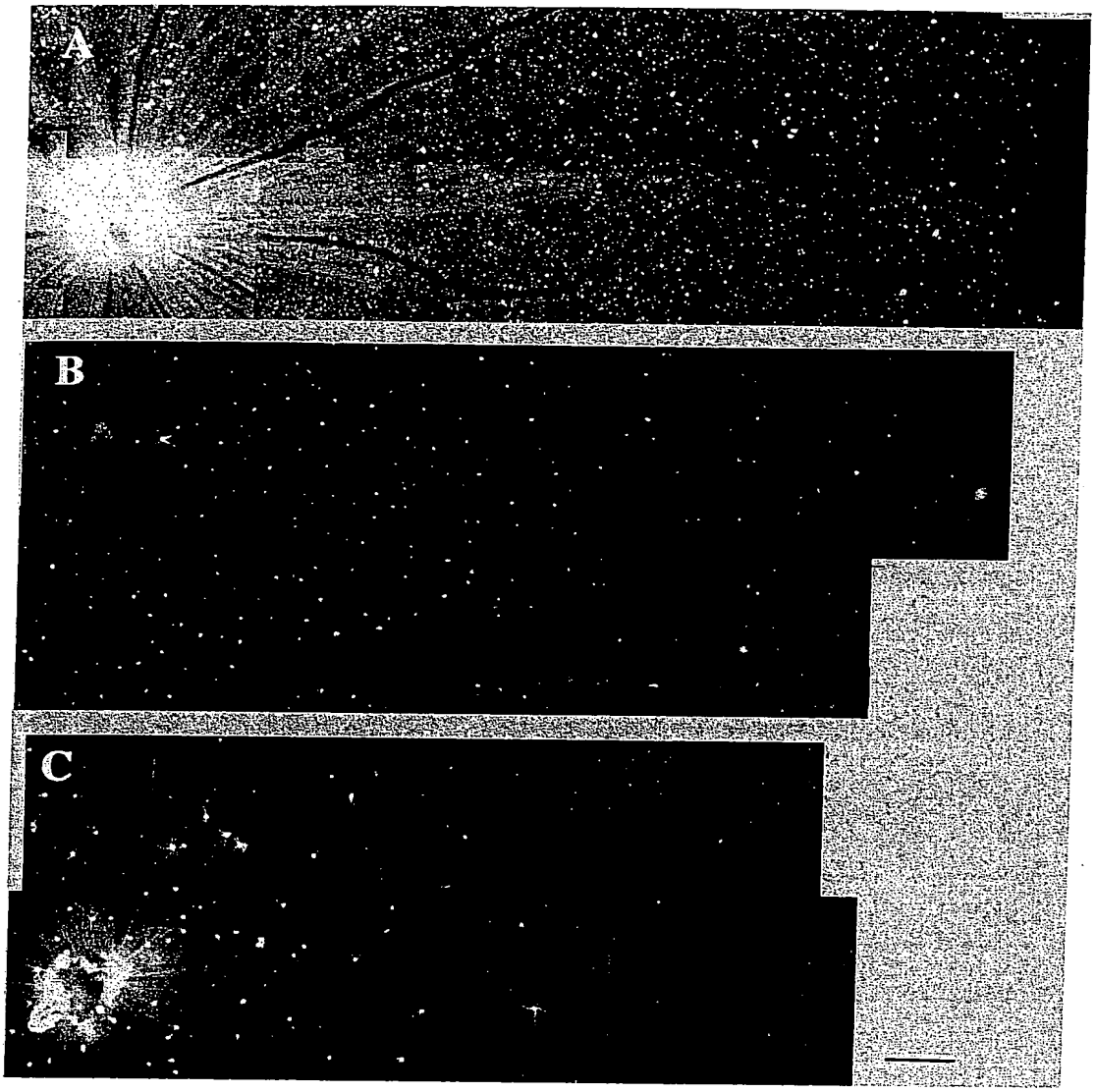

FIG. 3. Retrogradely labeled RGCs of noninjured and injured nerves following their secondary degeneration. The figure shows representative micrographs of normal retina (A), retina of injured optic nerve labeled 2 weeks after moderate crush injury (B), and retina of injured optic nerve labeled 4 weeks after moderate crush injury (C). Bar, 300 μm.

and so on, until eventually the process runs out of steam and stabilizes. Under our experimental conditions even a mild insult caused a relatively massive primary loss of neurons and extensive secondary degeneration, which stabilized between 1 and 2 months after the primary damage.

The results of this study may provide a plausible explanation for the progressive loss of neurons in chronic neuropathies even after the primary cause of the neuronal loss has been alleviated. Thus, for example, continuing loss of visual field has been observed in patients with glaucomatous neuropathy even after the intraocular pressure has been restored to normal (5, 25). The fact that in some patients the disease is arrested whereas in others it continues to progress despite the normalization of intraocular pressure might be a reflection of the severity of the damage existing at the time of initiation of antihypertensive treatment (5).

The topographical pattern of degeneration observed in the present study, i.e., loss of retinal ganglion cells at the periphery rather than at the center of the retina, is also reminiscent of that seen in glaucomatous neuropathy (22, 23), where topographical differentiation and death of neurons have been attributed to size differences. Cell size may be one of the criteria for preferential loss of peripheral ganglion cells in mature retina, on the assumption that the largest retinal ganglion cells are the most susceptible (20).

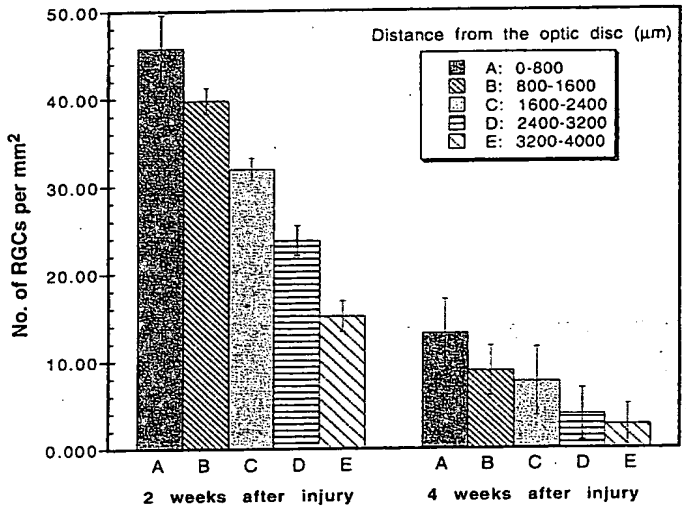

FIG. 4. Comparison of ganglion cell distribution in retinas of noninjured nerves as well as of injured optic nerves 2 and 4 weeks after moderate injury. Retinas were divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated by counting them in four different fields in each zone. The graph presents means ± SEM of four retinas examined 2 weeks or 4 weeks after the optic nerve was injured.

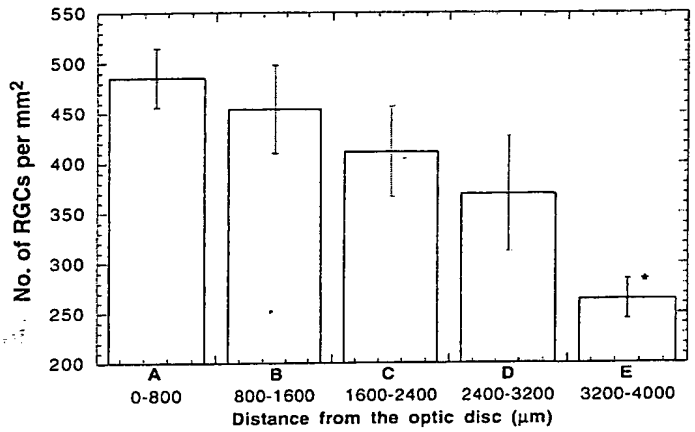

FIG. 6. Mechanical primary insult causes preferential loss of neurons whose ganglion cells are at the retinal periphery. Retinas were retrogradely labeled immediately following mild injury and were whole-mounted 1 week later. Retinas were divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated by counting them in four different fields per zone. The graph presents means ± SEM of four retinas. [ANOVA revealed a significant effect of the distance from the optic disk on the number of RGCs ($F = 4.28$; $P = 0.028$). *Significant difference ($P < 0.05$) vs the most central zone (zone A).]

It should be noted that although the crush injury inflicted in this study does not simulate human optic neuropathies from the point of view of etiology, it may resemble them in that the progression of nerve degeneration does not only depend on the nature of the primary cause. This further validates the use of a crush injury model for the study of secondary degeneration of neurons in chronic diseases such as glaucoma. Optic neuropathies involving raised intraocular pressure, which can be diagnosed and treated by pressure reduction, may thus benefit from neuroprotective therapy.

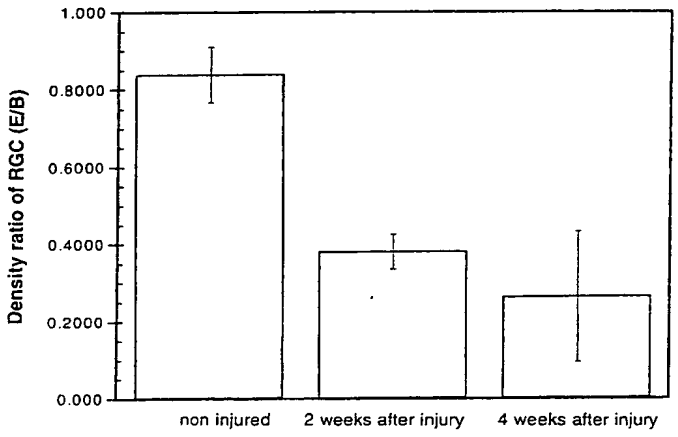

FIG. 5. Density of ganglion cells in the periphery relative to the center of the retina. The distribution of RGCs in normal retina and in retina of injured nerves 2 and 4 weeks after moderate optic nerve injury was determined by comparing the ratios of cell densities in zone B and zone E in each retina. The zones correspond to those shown in Fig. 4. The graph presents means ± SEM of the ratios obtained from two retinas of noninjured optic nerves and four retinas of optic nerves analyzed 2 and 4 weeks after injury.

The present study demonstrates the existence of secondary degeneration and strongly suggests that there is room for the development of neuroprotective therapy following acute or chronic injury where the primary cause can be identified and neutralized. Moreover, as secondary neuronal death is not only a self-perpetuating process but also a self-limiting one, the extent of nerve damage inflicted by acute trauma, or existing in chronic cases at the time of alleviation of the primary cause, will determine the extent of the secondary loss. It is important to emphasize that neuroprotection should be considered as the protection of intact neurons immersed in a degenerative environment rather than as the protection of cell bodies of injured axons; the latter, even if successful, does not lead to recovery of function, which is the criterion of effective neuroprotection. In selecting a strategy for neuroprotective therapy, it should be borne in mind that the hostile environment created by the degenerative neurons might contain several toxic elements; an appropriate therapy might therefore comprise a mixture of neuroprotective compounds of a broad spectrum of anti-degenerating mechanisms, or a compound with intracellular effect exerted downstream.

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